Regulation of G-proteins during chemotaxis in space and time

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Summary and discussion

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Summary and discussion

Chemotaxis, or regulated cell movement towards a chemical gradient, plays a role in a variety of processes in the human body, including wound healing, embryogenesis and metastasis (Jin, 2013; Zabel et al., 2015). The slime mold *Dictyostelium* is a commonly used model organism to study chemotaxis, for its amoeboid movement is similar to the movement of neutrophils and the signal transduction pathway is well conserved (Nichols et al., 2015). *Dictyostelium* cells use chemotaxis when scavenging for bacteria, mediated by the chemoattractant folic acid. When food is scarce the *Dictyostelium* cells will aggregate and form fruiting bodies containing spores. The aggregation process is mediated by chemotaxis to the self-produced chemo-attractant cAMP (Artemenko et al., 2014).

The chemotaxis pathway can be divided into four main modules consisting of G-protein coupled receptors (GPCRs), heterotrimeric G proteins, monomeric G proteins and finally myosin and actin from the cytoskeleton (Kortholt et al., 2011). Activation of the pathway begins with binding of a chemo-attractant to a GPCR, subsequently intracellular changes in the receptor lead to dissociation of the heterotrimeric G proteins Gα and Gβγ (Oldham and Hamm, 2008). These heterotrimeric G proteins activate downstream targets including Guanidine exchange factors (GEFs) which can activate monomeric G proteins such as Ras, Rac and Rap by stimulating the exchange of GDP for GTP. The monomeric G proteins activate many downstream signaling pathways which results in polymerization of filamentous actin (F-actin) at the front of the cell, and myosin mediated retraction of the back of the cell (Charest and Firtel, 2007; Nichols et al., 2015). This thesis has contributed in the understanding of regulation of the chemotaxis pathway, focusing on the many different ways of G protein regulation. The most important findings are discussed below and summarized in Figure 1.

Chemotaxis receptor regulation

Chapter 1 discusses the regulation of the chemotaxis pathway on receptor and heterotrimeric G protein level. Receptor activation is at the very start of the chemotaxis pathway, and regulation of both affinity and expression of receptors enables cells to be sensitive to a wide range of chemo-attractant concentrations (Fig. 1) (Junger, 2011; Song et al., 2006). Expression levels of receptors are regulated by transcription levels and by an internalization pathway upon receptor activation resulting in either resensitization by recycling of the receptor to the membrane or receptor degradation and desensitization (Dores and Trejo, 2014; Goodman et al., 1996; Hughes and Nibbs, 2018; Marchese, 2014; Marchese et al., 2003). Receptor affinity is regulated by phosphorylation of the receptor resulting in desensitization (Ali et al., 1999; Caterina et al., 1995). In human cells many receptors are known to bind and respond to more than one chemokine, and cells can express different chemoattractant receptors simultaneously (Hughes and Nibbs, 2018). This adds an extra layer of regulation where receptors can display differential responses to different chemoattractants, where receptors can be inhibited by antagonist binding, and where there can be a hierarchy to which chemoattractant a cell responds more strongly (Lämmermann and Kastenmüller, 2019; McDonald et al., 2010). Finally cells can accomplish a higher sensitivity range for chemo-attractants by degrading extracellular chemoattractants, creating a stronger gradient in the direct environment of the cell (Pálsson, 2009).

Heterotrimeric G protein regulation

Heterotrimeric G proteins consist of a Gα subunit and a Gβγ dimer. Dictyostelium has 8 different Gα genes and only one Gβγ gene, and of these different Gα proteins Gα4 associates with the folic acid receptor (faR) while Gα2 can bind to cAMP receptor 1 (cAR1). Gα exists in either an inactive GDP-bound form, or an active GTP-bound form. When Gα-GDP forms a heterotrimeric complex with Gβγ both are unable to activate downstream targets. Ligand binding to a GPCR results in intracellular conformational changes of the receptor, which promote the release of GDP from the Gα subunit. The empty pocket is quickly filled by GTP available from the cytosol, promoting disassociation of the Gα-GTP and Gβγ subunits. After some time GTP is hydrolyzed to GDP by the intrinsic Gα-associated GTPase activity, inactivating the heterotrimeric G proteins and enabling reassembly the Gα-GDPβγ complex (Greasley and Clapham, 2006). Heterotrimeric G protein activity is further regulated by GEFs, regulator of G protein signaling (RGS) proteins and guanine nucleotide dissociation inhibitors (GDIs). Non-receptor GEFs can amplify and extend heterotrimeric G protein signaling (Garcia-Marcos et al., 2009; Kataria et al., 2013). RGS proteins inhibit heterotrimeric G protein signaling by stimulating the intrinsic GTPase activity of Gα proteins, by antagonistic binding preventing binding to effectors and by promoting re-assembly of Gα-GDP and Gβγ upon GTP hydrolysis resulting in inactive protein complexes (Druey et al., 1996; Hollinger and Hepler, 2002). Lastly GDIs inhibit nucleotide exchange and trap Gα-GDP proteins in their inactive state (Fig. 1) (Kimple et al., 2002; Siderovski and Willard, 2005).

Connecting heterotrimeric and monomeric G protein signaling

The activation of heterotrimeric G proteins is proportional to the steepness of the gradient (Jin et al., 2000), whereas monomeric G proteins, such as Ras, Rac and Rap, are the first proteins in the chemotaxis pathway with significantly more activation at the leading edge compared to the back of the cell (de la Roche et al., 2004; Sasaki et al., 2004). Exactly how this symmetry breaking is achieved is not yet well understood, partly because only very few immediate effectors of Gα and Gβγ are known (Liu et al., 2016; Yan et al., 2012). In chapter 2 we identified Leucine rich repeat protein A (LrrA) through a proteomics screen as an interactor of both heterotrimeric and monomeric G proteins (Fig. 1). Pull down experiments indicate that G protein binding is nucleotide independent. Furthermore, Gα4 does not compete with Ras for binding to LrrA, indicating that LrrA can bind monomeric and heterotrimeric simultaneously. These results, in combination with the lack of any enzymatic domains in LrrA, suggest LrrA functions as a scaffold protein that connects monomeric and heterotrimeric G protein signaling pathways. *LrrA* deletion mutants have defects on almost all levels of the chemotaxis pathway. *LrrA*-null cells have less heterotrimeric G protein dissociation, elongated monomeric G protein responses, and a reduced F-actin polymerization upon cAMP stimulation. The knock-out cells fails to form fruiting bodies and *lrrA*-null is the first mutant known so far where addition of artificial cAMP pulses impedes rather than accelerates development. Finally deletion of *lrrA* results in more and smaller pseudopods and less efficient chemotaxis. We conclude that LrrA connects and integrates G protein signaling, and deletion results in spatial and temporal misregulation of G proteins and their downstream targets.

In future research it would be of great interest to find other interaction partners of LrrA. Possible binding partners include GAP proteins for Ras, Rap and Rac, which would explain the prolonged activation of these G proteins in the *lrrA*-null strain. To better understand

the pseudopod dynamics one could study the myosin dynamics in the LrrA mutant strains. Purification of the LrrA protein would also help with performing more detailed in vitro experiments, for example microscale thermophoresis and fluorescent polarization experiments to determine binding affinities for different interactors. These experiments could help determine whether and which binding partners work synergistically to achieve signal amplification, and which binding partners regulate localization. This thus would result in a more detailed model on the function of LrrA and may explain why deletion of LrrA leads to defects at all levels of chemotaxis signal transduction.

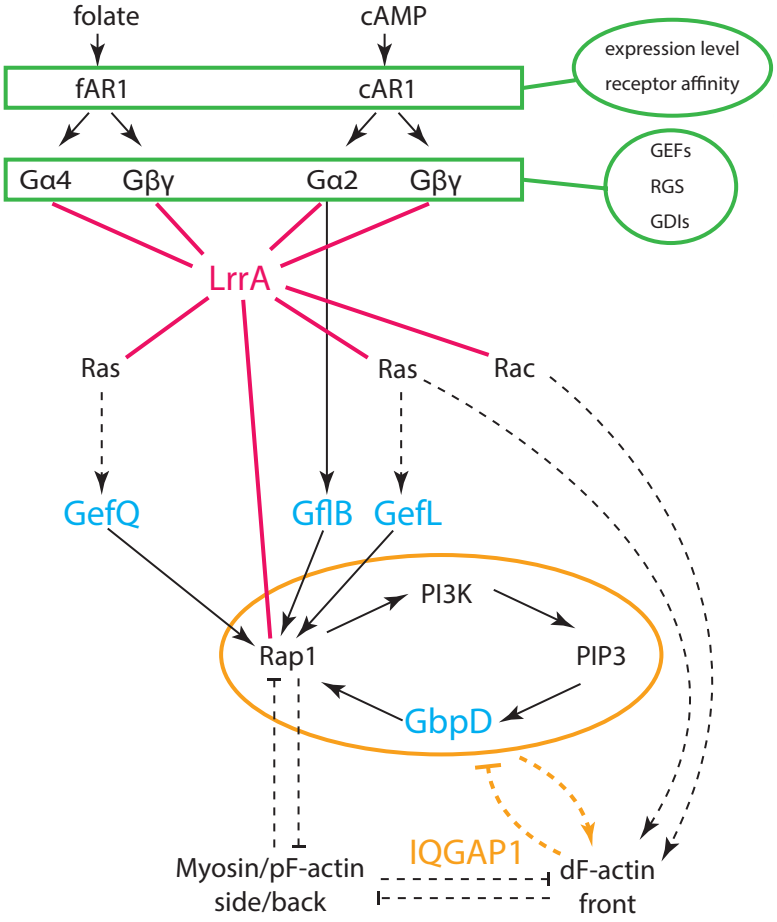

Figure 1. A schematic summary of the different parts of the chemotaxis pathway in *Dictyostelium* discussed within this thesis. In green the regulation of the chemotaxis receptors and heterotrimeric G proteins reviewed in chapter 1. In pink the scaffold protein LrrA connected to its interaction partners by lines, discussed in detail in chapter 2. In blue all four Rap1 GEFs, three of which have been investigated in chapter 3. In yellow the Rap1 amplification loop involving Rap1, PI3K, PIP3 and GbpD, and the IQGAP1 dependent inhibition of this loop by filamentous actin described in chapter 4. Solid lines represent direct interactions while dotted lines represent indirect interactions.

It is interesting to see that a scaffold protein can cause so many imbalances in a signaling pathway, but there are more examples found in literature. Another scaffold protein that is involved in the chemotaxis network in *Dictyostelium* is Sca1. Sca1 recruits two RasGEFs and phosphatase 2A (PP2A) to the leading edge in chemotaxing cells, and regulates Ras activation and actin dynamics (Charest et al., 2010). In mammalian cells the scaffold protein Scribble has been implied with even broader functions, regulating processes as diverse as polarity, adhesion, proliferation and neuronal signaling (Bonello and Peifer, 2019). It is becoming increasingly clear that scaffold proteins are important for spatial and temporal coordination of signaling pathways by assembling specific signaling components, regulating localization of signaling components, integrating positive and negative signals and preventing inactivation of signaling components (Good et al., 2011; Shaw and Filbert, 2009). Since scaffolds lack a clear enzymatic function and are implicated in a lot of protein-protein complexes they are often difficult to study. However, my study on LrrA shows that to completely understand the regulation of signaling pathways it will be crucial to characterize and identify additional scaffolding complexes.

Rap1 function and activation

Chapter 3 and 4 investigated the activation and regulation of the monomeric G protein Rap1. Rap1 is an important regulator of the actin cytoskeleton and is involved in many cellular processes including substrate adhesion, cytokinesis, phagocytosis and chemotaxis (Hilbi and Kortholt, 2017; Parkinson et al., 2009; Rebstein et al., 1993). Activation of G proteins is mediated by GEFs, while inactivation is achieved by Guanidine Activating Proteins (GAPs). Chapter 3 described the characterization of two new Rap1-specific GEF proteins, GefL and GefQ, the regulation of a previous identified GEF GbpD (Kortholt et al., 2006) and summarizes the functions of the four known Rap1GEFs including GflB (Fig. 1) (Liu et al., 2016).

GefQ is mainly involved in Rap1 responses in vegetative cells and during cytokinesis, and is important for substrate adhesion. The GefQ knockout strain has a severely decreased chemotaxis towards folate, while chemotaxis to cAMP is similar to wildtype cells (Chapter 3).

Conversely, *gefL*-null cells show normal folate responses, but have defects in cAMP responses and development. *GefL*-null cells have a normal RasG response, while the cAMP-mediated Rap1 response is severely decreased. Considering Rap1 activation requires intact RasC/G signaling (Bolourani et al., 2008) and the *gefL*$^{-}$ cells show a normal Ras response we suggest GefL functions downstream of Ras. During development slugs move poorly, and the spores from *gefL*-null fruiting bodies are less viable (chapter 3) (Wilkins et al., 2005).

GbpD is important for Rap1-mediated substrate adhesion and during vegetative growth (chapter 3) (Bosgraaf et al., 2005; Kortholt et al., 2006). Disruption of *gbpD* results in severely decreased adhesion, slightly lower chemotaxis towards a folate source and a lower activation of Rap1 at the cleavage furrow in dividing cells. In chapter 3 the regulation of GbpD is further elucidated, it describes how the GRAM domain binds the lipid PIP2 and is required for GbpD membrane localization. Both the GRAM and CNB1 domain are essential for GbpD function, while interaction between Rap1 and GbpD is dependent on PIP3. These results further support the previously described positive feedback loop where Rap1 activates PI3K resulting in PIP3 production, which activates GbpD which in turn activates Rap1 (Fig. 1) (Kortholt et al., 2006, 2010). In chapter 4 we suggest that this positive feedback loop functions as a driving force for Rap1 activation, while over-activation in any part of this loop results in excessive

Rap1 activation.

GflB is the fourth known GEF of Rap1 which activates Rap1 in a Gα2 dependent manner (Liu et al., 2016). GflB is mainly involved in chemotaxis towards cAMP, however recent studies suggest GflB also plays a role in cytokinesis and macropinocytosis (Inaba et al., 2017; Senoo et al., 2016).

Together these four GEFs can regulate Rap1 activity throughout the lifecycle of *Dictyostelium,* with GefQ and GbpD mainly regulating Rap1 activity in vegetative cells, and GefL and GflB regulating Rap1 during development. Though the regulation mechanism of GbpD and GflB are by now quite well understood, the regulation of both GefQ and GefL is not yet fully characterized.

Linking G protein activation and actin dynamics

To achieve cell movement *Dictyostelium* cells produce pseudopods at the front of the cell, while the uropod is retracted by myosin-mediated contraction of the back of the cell. A cell contains two types of F-actin polymers, the branched dendritic actin filaments (dF-actin) in the emerging pseudopod, and parallel actin filaments (pF-actin) that are associated with myosin filaments in the cortex at the side and the back of the cell (Beta, 2010; Davidson and Wood, 2016). In the cortex the formation of dF-actin is inhibited by pF-actin/myosin (Rotty et al., 2015; Suarez et al., 2015), explaining why cells with a weak cortex are less polarized and often extend pseudopods from the side and the back of the cell (Cha and Jeon, 2011; Lee et al., 2010). Dendritic actin polymerization is dependent on the Arp2/3 complex. Activation of Arp2/3 is mediated by the proteins WASP and WAVE/SCAR, which are activated by the small G protein Rac (Ibarra et al., 2005; Miki et al., 1998; Pollitt and Robert, 2009; Westphal et al., 2000). Both Rap1 and Ras, activated in the front of the cell, are known activators of Rac, and probably regulate actin activation through this pathway (Kortholt and van Haastert, 2008; Kortholt et al., 2010; Mun and Jeon, 2012). Furthermore, Ras and Rap mediated activation of the TORC2 and PKB pathway is thought to be involved in F-actin formation and chemotaxis, though the exact mechanism is unknown (Charest et al., 2010; Lee et al., 2005). Occasionally a new front is induced in the cell cortex by local Ras/Rap activation. At the new front disassembly of the pF-actin/myosin cortex is mediated by Rap1 and its downstream effector Phg2, which thereby enables branched dF-actin formation at the emerging front (Jeon et al., 2007a, 2007b). Interestingly, *lrrA*$^{-}$ cells generate many more pseudopods in both the front and the side of the cells compared to wildtype, suggesting a disruption of pF-actin/myosin in the cortex in the knock-out cells (chapter 2).

In chapter 4 we discovered that Rap1 does not only regulate dF-actin polymerization, vice versa actin polymers negatively regulate Rap1 activation. Incubation with the general F-actin inhibitor LatA results in uniform Rap1 activation. Rap1 inhibition by F-actin is dependent on both GbpD and IQGAP1 (Fig. 1). Our finding that F-actin does not inhibit Rap1 activation in either *gbpD*$^{-}$ cells or in cells treated with a PI3K inhibitor suggests that F-actin inhibits the entire positive feedback loop described above consisting of Rap1, PI3K, PIP3 and GbpD (Fig. 1). Therefore, there is an in-build mechanism where the outcome of Rap1 activation, i.e. F-actin formation, functions as a natural brake on Rap1.

LatA binds to free actin molecules and thus inhibits both pf-actin and dF-actin formation, making it unclear which F-actin mediates the inhibitory effect on Rap1. However, since the above described positive feedback loop generates excessive dendritic F-actin at the place of

Rap1 activation, it is more likely that dF-actin functions as the brake.

The inhibition by F-actin is dependent on IQGAP1 through a mechanism that is unknown (Fig. 1). IQGAP1 is a protein that is involved in separating the dF-actin pathways at the front from the pF-actin/myosin pathways at the side of a chemotaxing cells (Haastert et al., 2018; Kothari et al., 2019; Shannon, 2012). To explain the role of IQGAP1 in F-actin mediated inhibition of the positive feedback loop we propose two, non-exclusive, models: (1) IQGAP1 is part of the brake mechanism through direct binding to Rap1, possibly regulating similar to the mechanism IQGAP1 regulates Rac, by binding Rap1 and preventing binding to other regulators and effectors (Jeong et al., 2007). (2) The balance is shifted towards a myosin/pF-actin cytoskeleton in *iqgA*$^-$ cells which results in inhibition of Rap1 and the amplification loop (chapter 4). In the future it would be of great interest to discover at which point the feedback loop is inhibited, for example by investigating binding of actin to GbpD, and GbpD subunits. The mechanism of IQGAP1 mediated inhibition could be investigated by checking whether IQGAP1 competes for binding to Rap1 with Rap1 interactors using competitive pull downs, and by investigating whether myosin and actin dynamics are altered in *iqgA*$^-$ cells.

Conclusion and outlook

In conclusion it is clear that the chemotaxis pathway is strongly regulated at all levels of signal transduction. In this thesis many diverse ways of regulation have been discussed, including expression levels, ligand affinity, activation and inactivation of G proteins by GEFs, GAPs and GDIs, antagonistic binding by RGS, connecting signal components by scaffold proteins, signal amplification by positive feedback loops and natural brakes by negative feedback loops (Fig. 1). All these mechanisms can affect the timing and localization of different pathway components, ensuring both a robust but flexible signaling system. It is clear that the chemotaxis pathway consists of a very complex network with many interconnected feedback loops, which can complicate interpreting data. However by asking the right questions and performing detailed and dedicated experiments we're still able to gain more knowledge on the pathway.

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